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The HIV-1 integrase mutant R263A/K264A is two-fold defective for TRN-SR2 binding and viral nuclear import

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Background: Whether the TRN-SR2/HIV-1 IN interaction mediates the nuclear import of HIV is controversial.
Results: The replication defective HIV integrase mutant $IN^{R263A/K264A}$ displays a two-fold reduction in interaction with TRN-SR2 and PIC nuclear import.
Conclusion: The HIV-IN TRN-SR2 protein-protein interaction is important for HIV nuclear import
Significance: The IN/TRN-SR2 interaction interface is a potential target for future antiviral therapy.

Abstract

Transportin-SR2 (Tnpo3, TRN-SR2), a human karyopherin encoded by the *TNPO3* gene, has been identified as a cellular cofactor of HIV-1 replication, specifically interacting with HIV-1 integrase (IN). Whether this interaction mediates the nuclear import of HIV remains controversial. We previously characterized the TRN-SR2 binding interface in IN and introduced mutations at these positions to corroborate the biological relevance of the interaction. The pleiotropic nature of IN mutations complicated the interpretation. Indeed, all previously tested IN interaction mutants also affected reverse transcription (RT). Here we report on a virus with a pair of IN mutations, $IN^{R263A/K264A}$, that significantly reduce interaction with TRN-SR2. The virus retains wild-type reverse transcription activity but displays a block in nuclear import and integration, as measured by Q-PCR. The defect in integration of this mutant resulted in a smaller increase in the number of 2-LTR circles than for virus specifically blocked at integration by raltegravir or catalytic site mutations ($IN^{D64N/D116N/E152Q}$). Finally, using an eGFP-IN labeled HIV fluorescence-based import assay, the defect in nuclear import was corroborated. These data altogether underscore the importance of the HIV-IN TRN-SR2 protein-protein interaction for HIV nuclear import and validate the IN/TRN-SR2 interaction interface as a promising target for future antiviral therapy.

Introduction

Lentiviruses such as the human immunodeficiency virus type 1 (HIV-1) infect both dividing and non-dividing cells (1,2). Following reverse transcription, the pre-integration complex (PIC) composed of the viral DNA and a complex of viral and cellular proteins crosses the nuclear membrane via active nuclear import through the nucleopore (3). Nuclear entry precedes provirus establishment through stable insertion of the viral DNA into the host chromatin as catalyzed by HIV-1 integrase (IN) and constitutes a genuine bottleneck during HIV replication (4). As such it represents an interesting, yet unexploited target for antiviral therapy. Despite extensive research, the underlying mechanism of HIV nuclear import remains a contentious issue until today.

Several viral components, including capsid (CA), the central polypurine tract (cPPT), integrase (IN), matrix (MA) and viral protein R (Vpr) have been reported to play a role in nuclear import but no consensus has been reached (5-7). Nuclear import in the human cell is orchestrated by a plethora of karyopherins,

comprising both broad-spectrum and highly specialized transporters. Several of these have been suggested to take part in HIV nuclear import, in particular importin-α/β (8-10), importin-α3 (11) and importin-7 (12-15). In 2008, transportin-SR2 (TRN-SR2, transportin-3, Tnpo3), encoded by the *TNPO3* gene, was independently identified as a cellular cofactor of HIV in two genome-wide siRNA screens (16,17) and picked up as a specific binding partner of HIV IN (18). Using RNAi technology, the crucial role of TRN-SR2 in HIV replication was demonstrated (16-18).

Although the importance of TRN-SR2 for HIV replication is widely accepted and has been independently confirmed by different research groups, the exact mechanism of action remains unresolved (19-29). Because TRN-SR2 is a known karyopherin, involved in nuclear import of essential splicing factors, namely serine/arginine-rich proteins (SR proteins) and also certain non-SR proteins (30-33), it is a plausible candidate import factor for HIV.

IN catalyzes the insertion of the viral DNA into the host chromatin in two distinct steps. During 3'-processing the enzyme removes the 3'-terminal GT dinucleotide from both long terminal repeat (LTR) ends resulting in reactive 3'-OH groups. This reaction is believed to occur in the cytoplasm. The processed cDNA is then imported in the nucleus where a nucleophilic attack of the exposed 3'-OH groups on the target DNA phophodiester backbone and transesterification result in stable insertion of the viral DNA into the host chromatin. During HIV infection a minute fraction of the proviral cDNA is subjected to 1- and 2-long terminal repeat (LTR) circle formation in the nucleus, which can be monitored by Q-PCR (34). IN catalytic site mutations or the addition of integrase inhibitors during HIV infection reproducibly result in abortive integration and a steep increase in 2-LTR circles (34), whereas a decrease in 2-LTR circles is generally accepted to reflect a block in nuclear import (35). In line, a reduction in the number of 2-LTR circles was measured after RNAi-mediated TRN-SR2 depletion (18,20,21,36), suggesting a nuclear import defect. In a direct HIV nuclear import assay using IN-eGFP labeled PICs (18,37), we demonstrated a reduction of nuclear *versus* cytoplasmic PICs after TRN-SR2 knockdown (18), underscoring a role for TRN-SR2 in HIV nuclear import.

Although we and others have shown that TRN-SR2 directly interacts with HIV IN (18,19,27,38,39), the mechanism of action has been questioned (19,23,36,40,41). Especially reports on a set of HIV capsid mutations (e.g. N74D) that reduce the dependency of HIV replication on TRN-SR2 in single-round HIV infection assays, put forward an alternative hypothesis according to which TRN-SR2 exerts its role through direct interaction with the viral CA protein (19,23). However, in multiple round, spreading infection experiments, HIV carrying the N74D CA mutation remained sensitive to TRN-SR2 depletion (25). Possibly, these capsid mutations affect virus uncoating (25,42) and thus constitute a rate-limiting step prior to HIV nuclear import. Initially, the N74D capsid mutant was picked up as a resistance mutation against overexpression of a C-terminally truncated fragment of the mouse Cleavage and Polyadenylation Specificity Factor subunit 6 (mCPSF6-358) (43). CPSF6 is a cellular protein involved in splicing and polyadenylation of pre-mRNA that carries an RS domain at its C-terminus. Since CPSF6 binds HIV CA (40), it has been suggested that TRN-SR2 depletion might result in cytoplasmic accumulation of CPSF6, which, in turn, might perturb viral uncoating through CPSF6–CA binding, leading to an indirect block in nuclear import and restricted HIV replication (36,40,41). Still, the cytoplasmic accumulation of CPSF6 after TRN-SR2 knockdown is not always observed (36,41). In any case, the fact that a virus defective for interaction with CPSF6 (CA^{N74D}) remains sensitive to TRN-SR2 knockdown in a multiple-round replication experiment (25) suggests that cytoplasmic accumulation of CPSF6 is not a sole cause for the HIV replication deficit upon TRN-SR2-depletion.

These indirect lines of evidence do not unequivocally address the question whether the IN/TRN-SR2 interaction mediates nuclear entry. To study its role in HIV nuclear import a specific block in the IN/TRN-SR2 interaction is required. To uncouple the effect of TRN-SR2 knockdown from the possible cytoplasmic accumulation of CPSF6 or other cargoes, we decided to search for IN mutants specifically defective for TRN-SR2 binding. We previously characterized the TRN-SR2 interaction interface to identify key amino acids in the viral IN and

determined the hot spots for the interaction, namely the amino acids R262/R263/K264 and K266/R269 in IN (27). Independent confirmation of the role of R262, R263 and K264 was provided by Larue et al. (38).

We next decided to introduce specific IN interface mutants in NL4-3 backbones to study their effect on viral replication. However, since IN is an essential component of the nucleoprotein complex in the budding virus and of the reverse transcription complex (RTC), IN mutations can result in pleiotropic effects extending well beyond the integration step. It is well established that some IN mutants negatively affect reverse transcription (44,45). Out of the five amino acids identified as hot spots for interaction with TRN-SR2 (Figure 1), the K266A and R269A single point mutants as well as the double mutants R262A/R263A and R262A/K264A were reported before to negatively affect reverse transcription (44), confounding the detection of replication defects beyond reverse transcription, such as blocked nuclear import.

Here we describe a virus carrying an IN mutant with reduced affinity for TRN-SR2 (NL4-3 $IN^{R263A/K264A}$) that still displays wild-type reverse transcriptase activity. Demonstration of a nuclear import defect for this virus implies that the interaction between IN and TRN-SR2 mediates HIV PIC nuclear import.

Experimental procedures

Plasmids and Recombinant protein purification - Recombinant proteins were expressed in *E. coli* strain BL21-CodonPlus (DE3). Recombinant His_6-tagged HIV-1 IN (46) and recombinant GST-tagged TRN-SR2 (18) were purified as described previously. His_6-tagged HIV-1 IN was expressed using pINSD.His (obtained through the AIDS Research and Reference Reagent Program). The mutations in pINSD.His were introduced by site-directed mutagenesis using the *Kirsch and Joly* method (47). Megaprimers were ordered and used to perform a full round PCR on the pINSD.His plasmid. For the $IN^{R263A/K264A}$ mutation following megaprimers were used: pINSD-RK263toA-s: 5'CAATCATCACCTGCCATCTGTTTTCCATAATCCCT GATGATCTTTGCTGCTGCTCTTGGCACTACTTTTAT GTCACTATTATCTTG 3' and pINSD-RK263toA-as: 5' CAAGATAATAGTGACATAAAAGTAGTGCCAA GAGCAGCAGCAAAGATCATCAGGGATTATGGAAA ACAGATGGCAGGTGATGATTG 3'. All plasmid constructs used in this work were verified via sequence analysis.

AlphaScreen protein-protein interaction assay - The AlphaScreen binding assay was performed as described previously (27). In brief, proteins were all diluted to 5× working solutions in the assay buffer (25 mM Tris [pH 7.4], 150 mM NaCl, 1 mM $MgCl_2$, 2 mM DTT, 0.1% [v/v] Tween-20, and 0.1% [w/v] bovine serum albumin [BSA]). First, 10 µL of the GST-TRN-SR2 was pipetted into the wells, followed by 5 µL of a His_6-IN dilution series. The plate was sealed and left to incubate for 1 h at 4°C. Next, 10 µL of a mix of Ni^{2+} chelate acceptor and glutathione donor AlphaScreen beads (PerkinElmer) was added. Plates were then incubated for 1 h at 30°C and analyzed using an EnVision Multilabel Reader (PerkinElmer) according to the manufacturer's instructions. Points for which quenching of the signal occurred through excess of either binding partner ("hooking" as referred to by the supplier), were excluded from the final plot.

Integrase activity assays - 3'-processing activity was measured by detection of radioactively labeled products by denaturing gel electrophoresis and was performed as described previously (48). Briefly, 500 nM IN was incubated for 7.5 min at 37°C with 20 nM of the radioactively labeled oligonucleotide substrate (INT1, ^{32}P-5' GTGTGGAAAATCTCTAG CAGT 3'; INT2, 5'ACTGCTAGAGATTT TCCACAC 3'). The final reaction mixture contained 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.5, 5 mM dithiothreitol (DTT), 10 mM $MgCl_2$, 0.5% (v/v) polyethylene glycol 8000, 0.5% BSA and 15% dimethylsulfoxide (DMSO). The reactions were stopped by the addition of 50% vol formamide. Samples were loaded on a 15% denaturing polyacrylamide/urea gel. The extent of 3'-processing was measured based on the respective intensity of -2 bands relative to the total radioactivity present in the lane. These data were determined using the OptiQuant Acquisition and Analysis software (Perkin Elmer Corporate). Strand-transfer activity was measured with a quantitative ELISA as described previously (49).

Cell culture - HeLaP4 cells (obtained from the NIH Reagent Program) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 50 µg/ml gentamicin (Gibco, BRL) and 5% fetal calf serum (International Medical, Belgium). Cells were incubated at 37°C and 5% CO2 in a humidified atmosphere. Two different HeLaP4 cell lines stably knocked down for importin-α3 were generated by transduction with two different lentiviral vectors expressing shRNAs targeting the importin-α3 mRNA, called sh93 and sh97 (Sigma, clone-id NM_002268.3-1814s1c1 and NM_002268.3-605s1c1). A control cell line was established using a control vector expressing a scrambled shRNA referred to as shSCR (Sigma, product number SHC002). These lentiviral transfer plasmids were a kind gift from Dr. R. Hoeben (Leiden University Medical Center, The Netherlands). The shRNA expressing transfer plasmids are based on the pLKO.1 plasmid (Sigma) containing a puromycin resistance cassette. After transduction cells were selected and grown in DMEM as described above with the addition of 1 µg/ml puromycin. MT-4 cells (obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Dr. Douglas Richman) were grown in RPMI 1640 (GIBCO 461 BRL) supplemented with 12% FCS and 50 µg/ml gentamicin. Human peripheral PBMCs were purified from fresh buffy coats of anonymous voluntary donors using Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) following the manufacturer's protocol. Subsequently, PBMCs were maintained and stimulated in RPMI 1640 supplemented with 15% FCS, 20 U/ml IL-2 and 10.

Western blotting - For western blotting, protein concentrations of 1% SDS whole cell extracts were determined using the BCA protein assay (Thermo Scientific Pierce) and after separation by SDS-PAGE 25 µg of each extract was electroblotted onto polyvinylidene difluoride membranes. Membranes were probed with monoclonal antibodies against importin-α3 (1:500 dilution) (ab6039, abcam) and β-actin (1:5000 dilution) (A5441 Sigma) antibodies were used to confirm equal loading. Detection was performed using horseradish peroxidase-conjugated antibodies (Dako) and chemiluminescence (Pierce ECL+, Thermo scientific).

Viruses and viral vectors - The viral molecular clones pNL4-3 and pNL4-3.Luc.RE- were obtained through the AIDS Research and Reference Reagent Program. Mutations in pNL4-3 and pNL4-3.Luc.RE- were introduced by site-directed mutagenesis using the *Kirsch and Joly* method (47). Briefly, a sequence comprising the integrase coding region, amplified by PCR using pNL4-3 as a template, was cloned into pCR4-Topo (Invitrogen). This plasmid was then used to perform *Kirsch and Joly* site-directed mutagenesis using the pINSD-RK263toA-s and pINSD-RK263toA-as primers. The mutated IN sequence was then cloned back into pNL4-3 or pNL4-3.Luc.RE- using two unique restriction sites (AgeI and PflMI).Viruses were produced by polyethylenimine (PEI)-mediated transfection of 293T cells using 20 µg of the viral molecular clone pNL4-3 per 10 cm dish, for vectors an extra 5 µg of the pMD.G plasmid encoding the vesicular stomatitis virus glycoprotein (VSV-G) (50) was added. Two and three days post transfection, the supernatant was harvested, filtered through 0.22 µm pore-size syringe filters (Sartorius), concentrated by centrifugal filtration using Vivaspin concentrators (Millipore) and treated with DNase I (Roche) for 1 h at 37°C. For the single round experiments HeLaP4 cells (10^4 cells) stably depleted of importin-α3 and control cells were seeded into 96-well plates. One day later cells were infected in triplicate with 2 dilutions (21000 and 7000 pg p24) of VSV-G pseudotyped single-round HIV-1 virus in a total volume of 100 µl per well. Each well was lysed 72 h after infection using 50 µl of lysis buffer (50 mM Tris/HCl, pH 7.3, 200 mM NaCl, 0.2% NP40, 5% glycerol) and was analyzed for firefly luciferaseactivity (ONE-Glo™, Promega, Belgium) according to the manufacturer's protocols. Chemiluminescence was measured with a Glomax luminometer (Promega, Belgium). The protein concentration of each sample was determined (BCA Protein Assay Kit, Thermo Scientific Pierce) and readouts were normalized to 1 µg total protein. For the breakthrough experiments HeLaP4 cells (3.10^4 cells), MT-4 cells (10^5 cells) or PBMCs (10^6 cells) were infected with virus amounting to 30,000, 50 or 20,000 pg of p24, respectively. Starting three days after infection, supernatants were sampled daily.

Q-PCR quantification of HIV DNA species - 10^6 MT-4 cells were infected with 10^6 pg p24 of virus. Inhibitors, if present, were 2 µM zidovudine (AZT) or 0.6 µM raltegravir (Ral). At 2 h post-infection, supernatants were replaced with fresh Dulbecco's Modified Eagle Medium (DMEM) medium containing DMSO, AZT or Ral. Ritonavir (4 µM) was added to all samples to restrict replication to a single round. At distinct time points after infection, cells were lysed by the addition of a 10 mM Tris (pH 8.0) buffer containing 0.02% triton-X-100, 0.02% sodium dodecyl sulphate (SDS), 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.8 mg of proteinase K/ml and incubated for 1 h at 56°C. Q-PCR was performed directly on the lysates. A specifically designed set of TaqMan probe and primers was used to quantify the amount of each specific HIV-1 DNA form in the cell lysate as described previously (34,51,52). For the quantification of reverse transcripts (total HIV-1 DNA), the forward primer 5′-ATCAAGCAGCCATGCAAATGTT-3′, the reverse primer 5′-CTGAAGGGTACTAGT AGTTCCTGCTATGTC-3′, and the probe 5′-(FAM)-ACCATCAATGAGGAAGCTGCAGA ATGGGA-(TAMRA)-3′ were used. For the quantification of 2-LTR circles, the forward primer 5′-GTGCCCGTCTGTTGTGTGACT-3′, the reverse primer 5′-CTTGTCTTCTT TGGGAGAGAATTAGC-3′, and the probe 5′-(FAM)-TCCACACTGACTAAAAGGGTCTGA GGGATCTCT-(TAMRA)-3′ were used. Quantification of integrated proviruses was performed by nested *Alu*-PCR, as described previously (51). For the first-round, the forward primer, 5'-CTAACTAGGGAACCCACTGCT TA-3' and the reverse primer, 5'-TGCTGGGATTACAGGCGTGAG-3' were used. The reaction contained 2 mM $MgCl_2$, 250 µM dNTPs, 400 nM primers, 1.25 U Taq DNA polymerase (Fermentas), 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$ and 0.01% Tween 20. For the Q-PCR, the forward primer, 5'-AGCTTGCCTTGAGTGCTTCAA-3', the reverse primer, 5'-TGACTAAAAGGGTCTG AGGGATCT-3' and the probe 5'-(FAM)-TTACCAGAGTCACACAACAGACGGGCA-(TAMRA)-3' were used. In each sample RNase P DNA was quantified as the endogenous control (Applied Biosystems). For each real-time PCR analysis, a standard curve was generated using dilutions of a representative sample. No-template controls (no DNA added to the PCR mixture) were run with each experiment. Reactions contained 1× iQ supermix (Biorad), 300 nM forward and reverse primer and 200 nM probe. After initial incubations at 95°C for 5 min, 50 cycles of amplification were carried out at 95°C for 10 s, followed by 30 s at 55°C. Reactions were analyzed using the LightCycler®480 (Roche).

PIC Nuclear Import Assay – The PIC nuclear import assay was performed with adaptations to the previously described protocol (37). 293T cells were transfected with 15 µg pVpr-IN-eGFP (Vpr and IN were cloned into the pEGFP-N1 vector from Clontech Laboratories), 15 µg pNL4-3.Luc.R-.E- (obtained from the AIDS Reference and Reagent Program), and 5 µg of the pMD.G plasmid encoding the vesicular stomatitis virus glycoprotein (VSV-G) (50). Supernatant was collected after 48 h, filtered through a 0.45 µm pore-size filter (Sartorius), and then concentrated by ultracentrifugation at 27500 rpm for 1 h 45 min. p24 was measured with the INNOTEST HIV Antigen mAb kit of Innogenetics. A viral inoculum of 3.10^6 pg p24 was used to infect 30,000 HeLa-P4 cells. Five hours after infection, cells were fixed with 2% paraformaldehyde. The nuclear lamina was visualized by staining with a monoclonal anti-lamin A/C antibody (Santa Cruz, sc-7292) and a secondary goat anti-mouse IgG Alexa-Fluor 633. Three-dimensional stacks of fixed cells were acquired with a Zeiss LSM510 multiphoton confocal microscope (Cell Imaging Core CIC, University of Leuven) equipped with a Plan-Apochromat 63x/1.4 Oil DIC objective. The Z-step size was 0.3 µm. Ar and HeNe laser lines were used for EGFP and Alexa-633 excitation. A sequential image acquisition was used. The quantification of the PICs was performed using a homemade MatLab routine (The MathWorks, Inc.). In brief, after adequate image processing, points were automatically detected using an intensity threshold. A fluorescent spot was assigned as a PIC if at least two connecting pixels are above the threshold and if the fluorescent signal is present in at least two consecutive frames (z planes). PICs were classified as cytoplasmic, in the nucleus or at the nuclear membrane based on the nuclear lamin staining.

Results

Selection of mutants defective for IN/TRN-SR2 interaction to study nuclear import of HIV

We previously characterized the TRN-SR2 interaction interface to identify key amino acids in viral IN. Next we introduced these specific IN interface mutants in NL4-3 backbones to study their effect on viral replication. Out of the five amino acids identified as hot spots for interaction with TRN-SR2 (Figure 1), the K266A and R269A single point mutants as well as the double mutants R262A/R263A and R262A/K264A were reported before to negatively affect reverse transcription (44), confounding the detection of replication defects beyond reverse transcription, such as a possible block in nuclear import. Therefore we tested the remaining mutants, namely the single point mutants R262A, R263A, K264A and the double point mutant R263A/K264A for their binding to TRN-SR2 using AlphaScreen (Figure 1B). AlphaScreen is a bead-based protein-protein interaction assay in which binding of the proteins results in light emission (AlphaScreen signal). The single point mutants R262A, R263A and K264A did not affect TRN-SR2-binding (data not shown) whereas $IN^{R263A/K264A}$ showed a reduction in AlphaScreen signal. We therefore performed an outcompetition AlphaScreen experiment to quantify the change in affinity to TRN-SR2 compared to wild type (WT) IN. We outcompeted the binding between GST-TRN-SR2 and His-tagged WT IN or $IN^{R263A/K264A}$ with untagged CTD of IN and calculated the IC_{50} of IN CTD in three independent experiments each performed in duplicate. The IC_{50} for outcompetition with IN CTD, was 172 nM (CI95% = 24.6 to 237.9 nM) for WT IN and 74 nM (CI95% = 45.95 to 119.5 nM) for $IN^{R263A/K264A}$, pointing to a significant 2.3-fold lower affinity of mutant IN for TRN-SR2 than WT IN $p = 0.0154$ (Figure 1B).

Next we compared the catalytic activities of WT IN, the double mutant $IN^{R263A/K264A}$ and the enzymatically dead IN^{D64A} (53). The 3'-processing activity was severely affected, as $IN^{R263A/K264A}$ only showed residual activity in comparison with WT IN (Figure 1C). Both $IN^{R263A/K264A}$ and IN^{D64A} were defective for strand transfer as determined in a quantitative ELISA assay (Figure 1D). The lack of catalytic activity of the $IN^{R263A/K264A}$ mutant however did not hamper the analysis of reverse transcription or nuclear import as these steps precede integration. In a previous study we showed using size exclusion chromatography that the R262A/R263A/K264A substitutions in IN do not affect IN oligomerization (27). We hence decided to study $IN^{R263A/K264A}$ in the context of the virus.

Characterization of HIV-1 NL4-3 virus carrying $IN^{R263A/K264A}$

The $IN^{R263A/K264A}$ mutations were inserted into a molecular clone of HIV (pNL4-3). Mutant and WT virus were harvested after transient transfection of 293T cells and normalized for p24 level or reverse transcriptase activity. HeLaP4, MT-4, and peripheral blood mononuclear cells (PBMC's) isolated from donor blood samples were infected with equal amounts of WT or $IN^{R263A/K264A}$ virus. Viral replication was monitored by sampling the supernatant as long as cells were viable. As expected from the reduced IN activities, $IN^{R263A/K264A}$ virus was completely replication-deficient in primary cells (Figure 1E) as well as in HeLaP4 and MT-4 cells (data not shown).

In 2010, a potential role of importin-α3 in HIV nuclear import was reported (11). Later the IN amino acids R263 and K264 were claimed to be involved in the binding to importin-α3(54). We therefore analyzed the effect of importin-α3 knockdown on HIV replication. We generated two stable importin-α3 knockdown cell lines (sh93 and sh97) and infected those with HIV NL4.3-Fluc. Potent knockdown of importin-α3 (95% and 99%, respectively) did not significantly reduce HIV infection compared to control cells (shSCR) (Figure 2). In comparison a 70% knockdown of TRN-SR2 resulted in a 10-fold reduction of HIV infection (18). Due to the uncertain role for importin-α3 in HIV-1 infection, the $IN^{R263A/K264A}$ mutant was used to study the specific role of the IN/TRN-SR2 interaction during HIV nuclear import.

To pinpoint at which step viral replication of the $IN^{R263A/K264A}$ virus was blocked, we monitored formation of viral DNA intermediates during single-round HIV-1 infection by Q-PCR for late reverse transcripts, integrated copies and 2-LTR circles. To control for the 3'-processing deficiency of $IN^{R263A/K264A}$, we not only compared the mutant to WT virus but also to the NL4-3 $IN^{D64N/D116N/E152Q}$ mutant virus (55). We generated this triple mutant, which has a mutated catalytic triad and lacks all enzymatic activity, as a more stringent control than the D64A mutant used previously (53).

Late reverse transcripts were measured by Q-PCR at 4, 8, 10 and 24 hours post-infection (Figure 3A and B). The HIV NL4-3 $IN^{R263A/K264A}$ mutant produced a similar number of reverse transcripts (taken at 8 and 10 hours post-infection) as WT virus whereas the $IN^{D64N/D116N/E152Q}$ mutant produced less reverse transcripts (93.6 ± 8.8% for NL4-3 $IN^{R263A/K264A}$ $p = 0.369$ and 68.9 ± 4.5% for NL4-3 $IN^{D64N/D116N/E152Q}$ $p = 0.01$). Conversely, the chain terminator AZT effectively blocked reverse transcription of all viruses whereas the strand transfer inhibitor raltegravir (Ral) did not affect reverse transcription (Figure 3A and B). Next, integrated viral DNA was measured by Q-PCR at 48 hours post-infection. The number of integrated copies of WT NL4-3 was effectively reduced by addition of AZT or Ral. Even though reverse transcription activities were comparable to WT virus (gray bars), both NL4-3 $IN^{R263A/K264A}$ mutant virus as well as $IN^{D64N/D116N/E152Q}$ displayed a block in integration compatible with their loss of 3'-processing and strand transfer activities (Figure 3C and D). Taken together these experiments indicate that these mutants both affect a step after RT, either during nuclear import or integration.

Next, we measured the formation of 2-LTR circles as an indirect measure for nuclear import at 24 hours post-infection (35). As expected (56) a 10–fold increase in the number of 2-LTR circles was evidenced when Ral was added during infection with WT NL4-3 (gray bars) (Figure 3E and F). Due to the block in integration the virus NL4-3 $IN^{D64N/D116N/E152Q}$ also produced increased numbers of 2-LTR circles, reaching comparable levels as WT NL4-3 supplemented with Ral. No further increase upon addition of Ral was observed as expected for a catalytically dead mutant (Figure 3F). The Q-PCR data on the 2-LTR circles of the $IN^{R263A/K264A}$ mutant require careful analysis since a block of integration will typically result in increased levels of 2-LTR circles but a concomitant block in nuclear import will lead to reduced 2-LTR circle formation (18,20,21,36). Although the number of 2-LTR circles of the $IN^{R263A/K264A}$ mutant was 2-fold higher than that of WT virus (Figure 3E), this increase was much lower than expected for a virus affected at the integration step (on average 10-fold higher) (Figure 3F). Even though the different viral preps were normalized for p24 and showed a comparable extent of reverse transcription, 2-LTR circles of the $IN^{R263A/K264A}$ mutant increased to levels that were 2.5-fold lower ($p = 0.0004$) than those of the catalytic dead mutant. Upon addition of Ral to WT virus a 10-fold increase in 2-LTR circles was measured ($p = 0.0002$), which is typically seen with a block in integration. These data indicate that NL4-3 $IN^{R263A/K264A}$ is also affected at a step following reverse transcription and before integration, thus most presumably at the nuclear import step.

Nuclear import defect of NL4-3 $IN^{R263A/K264A}$ virus

To confirm the effect of $IN^{R263A/K264A}$ on HIV nuclear import we finally performed a PIC nuclear import assay (37) (see materials and methods for more details). We produced two distinct IN-eGFP labeled viruses, one with WT IN and the other with the $IN^{R263A/K264A}$ double mutant. Five hours after infection the fluorescently labeled PICs were visualized using confocal microscopy (Figure 4A). The ratio of nuclear PICs over cytoplasmic PICs (percentage nuclear PICs) is a measure of nuclear import (18). To analyze HIV carrying $IN^{R263A/K264A}$, two independent experiments, with distinct productions of both the WT and the $IN^{R263A/K264A}$ mutant virus, were performed. Cytoplasmic and nuclear PICs were counted (Figure 4B). The ratio of nuclear *versus* cytoplasmic PICs, was calculated for both independent experiments. The difference between the distributions of the % nuclear PICs for WT and $IN^{R263A/K264A}$ mutant PICs was highly significant (WT, $n = 51$ cells; $IN^{R263A/K264A}$, $n = 49$ cells; $p = 0.0054$, Mann-Whitney test) and (WT, $n = 41$ cells; $IN^{R263A/K264A}$, $n = 44$ cells; $p = 0.0095$, Mann-Whitney test) (Figure 4B). Combining the data sets of the two individual experiments the *p*-value further decreased to 0.0001 (WT, $n = 92$ cells; $IN^{R263A/K264A}$, $n = 93$ cells). The mean % nuclear PICs calculated with 95% confidence interval was 4.1% for the WT (95% confidence interval, CI [3.5%; 4.7%]) and 2.4% for $IN^{R263A/K264A}$ (95% CI [2.1%; 2.8%]). When the percentage of cells that maximally contain a specific number of nuclear PICs was plotted, 50 % of the $IN^{R263A/K264A}$ mutant virus infected cells contained 0 to 1 % nuclear PICs, whereas 50 %

of the WT virus infected cells contained 0 to 3 % nuclear PICs, in line with a reduced nuclear import for HIV $IN^{R263A/K264A}$ and corroborating the Q-PCR results.

In a following experiment we evaluated the combined effect of TRN-SR2 depletion and interface mutants on PIC nuclear import. Stable TRN-SR2 depletion (shTR3) resulted in a significant reduction in nuclear PICs compared to control cells (shSCR) (WT in shSCR cells, n = 55 cells; WT in shTR3 cells, n = 49 cells; *p* = 0.0023, Mann-Whitney test) (Figure 4C) as shown previously after transient depletion of TRN-SR2 (18). The mean % nuclear PICs calculated with 95% confidence intervals was 2.2% for the control cells (95% confidence interval, CI [0.8%; 3.6%]) and 0.5% for TRN-SR2 knockdown cells (95% CI [0.3%; 0.7%]). We plotted the percentage of cells that maximally contain a specific percentage of nuclear PICs. For example, 95 % of the TRN-SR2 knockdown cells contain 0 to 2 % nuclear PICs, whereas 95 % of the control cells contain 0 to 5 % nuclear PICs. In parallel the $IN^{R263A/K264A}$ virus was tested both in shSCR cells, n = 50 cells and in shTR3 cells, n = 50 cells. Also in this experiment nuclear import of the WT virus was significantly higher than that of the $IN^{R263A/K264A}$ mutant virus; p = 0.0167. In shTR3 cells data for the WT virus (mean % nuclear PICs = 0.5) were not statistically different from data for the $IN^{R263A/K264A}$ mutant virus in shSCR cells (mean % nuclear PICs = 0.7); p = 0.5836. For the $IN^{R263A/K264A}$ mutant virus in shTR3 cells the mean % nuclear PICs was calculated with 95% confidence intervals as 0.2% (95% confidence interval, CI [0.1%; 0.3%]) which was significantly different from the $IN^{R263A/K264A}$ mutant virus in shSCR cells; p = 0.002 indicating an additive effect of TRN-SR2 depletion and interface mutations.

Discussion

In 2008 we identified TRN-SR2 as an interaction partner of HIV-1 integrase by yeast-two-hybrid-screening (18). Using RNAi technology, we could demonstrate the crucial role of TRN-SR2 during HIV replication both in dividing and non-dividing cells (18). Independently, two genome-wide siRNA screens also identified TRN-SR2 as a crucial cofactor for HIV replication (16,17). While this paper was in revision two papers were published revealing the crystal structure of TRN-SR2 (57,58). The TRN-SR2 mutant, D750A/D751A, was defective for interaction with CPSF6 and proved unable to support HIV-1 infection, although reportedly retaining WT level of HIV IN and ASF/SF2 binding (57). In our hands $TRN\text{-}SR2^{D750A/D751A}$ displayed a slightly reduced affinity for IN but was strongly impaired for binding to unphosphorylated ASF/SF2, suggesting that this mutant is generally reduced in its cargo binding capacity (data not shown).

Although indirect evidence suggests that the IN/TRN-SR2 interaction mediates HIV nuclear import, we thus needed to specifically block this interaction to corroborate its role in HIV nuclear import. To uncouple the effect of TRN-SR2 knockdown from the possible cytoplasmic accumulation of CPSF6 or other cargoes, we decided to search for IN mutants selectively defective for TRN-SR2 binding. We previously characterized the TRN-SR2 interaction interface to identify key amino acids in IN and determined as hot spots the amino acids R262/R263/K264 and K266/R269 (27). Independent confirmation of the role of R262, R263 and K264 was provided by Larue et al. (38).

Here we aimed to insert specific IN interface mutants in HIV molecular clones to study their effect on viral replication. However, out of the five amino acids identified as hot spots for interaction with TRN-SR2, the K266A and R269A single point mutants as well as the double mutants R262A/R263A and R262A/K264A were reported to negatively affect reverse transcription (44), confounding the detection of replication defects beyond reverse transcription, such as blocked nuclear import. Recently Li et al. reported on the IN^{K264E} mutant that is defective for 3' processing and strand-transfer in typical *in vitro* assays, but still displayed concerted integration activity. Unfortunately, reverse transcription by HIV-1 IN^{K264E} was reduced by 3- to 16-fold, obscuring further analysis (59). Therefore we tested the remaining alanine mutants, namely the single point mutants R262A, R263A, K264A and the double point mutant R263A/ K264A for their binding to TRN-SR2. The single point mutants R262A, R263A, K264A did not affect TRN-SR2-binding whereas the double point mutant R263A/ K264A inhibited TRN-SR2 binding 2.3-fold (Figure 1B). We therefore selected the

$IN^{R263A/K264A}$ mutant that interacts less with TRN-SR2 and does not perturb reverse transcription, allowing us to study the role of the TRN-SR2/IN interaction in the nuclear import of the PIC. In the past several attempts have been made to define potential NLSs in IN. Two stretches of amino acids, ^{211}KELQKQITK^{219} and ^{261}PRRKAK^{266} , were described as a potential bipartite NLS (8). Our analysis of the IN/TRN-SR2 interface has allowed us to confirm the following sets of amino acids in IN: R262/R263/K264 and K266/R269 (27). Lysine 264 was found to be acetylated by p300 by Cereseto et al. (60). Importin 7 and importin-α3 have both been implicated as nuclear import factors for HIV through a direct interaction with IN (14,54). Although importin 7 was later invalidated as HIV-1 nuclear import factor (11), R263 and K264 were reported as interacting amino acids. Previously the same two IN amino acids were implicated in the interaction between IN and importin-α3 (54). In our hands potent knockdown of importin-α3 did not affect HIV replication bringing into question the prior claims for a significant role for importin-α3 in HIV-1 replication (Figure 2). To assess the impact of a deficient IN/TRN-SR2 interaction on the nuclear import of HIV, the $IN^{R263A/K264A}$ interface mutations were inserted into a molecular clone of HIV (pNL4-3) resulting in a replication-deficient virus. As in vitro analysis of the mutant integrase has evidenced a strong defect in catalytic activity, it was expected that the mutant virus does not integrate and is replication defective. To pinpoint at which step replication is inhibited, we performed Q-PCR analyses on the different viral DNA intermediates. In parallel we investigated the $IN^{D64N/D116N/E152Q}$ catalytic triad mutant virus to control for the effect of defective 3'-processing on the formation of the viral DNA species. It is well known that a block in integration leads to an accumulation of 2-LTR circles in the nucleus (34). Indeed, both addition of Ral during infection with WT NL4-3 and infection with NL4-3 $IN^{D64N/D116N/E152Q}$ virus resulted on average in a 13-fold increase in 2-LTR circles. Remarkably, infection with NL4-3 $IN^{R263A/K264A}$, which is also defective for integration, only resulted on average in a 6-fold increase in 2-LTR circles. Compared to WT NL4-3, NL4-3 $IN^{R263A/K264A}$ displayed an increased number in 2-LTR circles, due to its defective integration. However in comparison with the number of 2-LTR circles for WT NL4-3 supplemented with Ral and NL4-3 $IN^{D64N/D116N/E152Q}$ on average a relative 2-fold decrease was measured ($p = 0.01$), suggesting a defect in nuclear import. This reduction may still be an underestimate of the true effect, since lack of IN strand transfer activity (as observed for $IN^{R263A/K264A}$ in vitro) is associated with an increase in 2-LTR circle formation due to aborted integration that may counteract the reduction in circles resulting from reduced PIC import.

Although well-established, Q-PCR analysis of 2-LTR circles is an indirect way to measure nuclear import of HIV. Therefore we additionally performed a cellular PIC nuclear import assay, which allows us to directly follow HIV PICs in infected cells (18,37). In this assay IN-eGFP is *trans*-incorporated into viral particles through Vpr fusion, enabling visualization by confocal microscopy. The ratio of nuclear to cytoplasmic PICs allows for direct quantification of nuclear import. Whereas 2-LTR accumulation is typically measured at 24 h after infection prior to dilution by cell division, nuclear PICs are imaged by fluorescence at 5 h post infection since integration is believed to dissassemble the PICs. Albanese et al. performed a kinetics experiment to quantify the number of nuclear PICs at different time points from 3 to 24 hours after infection and showed that the maximum number of intranuclear PICs was indeed reached after 6 hours (37).

This PIC import assay further corroborated the block in nuclear import observed with the $IN^{R263A/K264A}$ virus in comparison with WT virus. The reduction in nuclear PICs of the $IN^{R263A/K264A}$ virus was similar to the reduction seen after TRN-SR2 depletion. When analyzing $IN^{R263A/K264A}$ in shTR3 cells the combination of both partial TRN-SR2 depletion and 2-fold reduced IN-TRN-SR2 interaction resulted in an additive effect on the inhibition of nuclear import.

The on average 2-fold reduction in nuclear PICs and the 2-fold drop in 2-LTR formation and was perfectly in line with the 2.3-fold lower binding affinity of $IN^{R263A/K264A}$ for TRN-SR2 compared to WT IN.

We describe here how the NL4-3 virus, carrying $IN^{R263A/K264A}$ which is deficient for interaction with TRN-SR2, is blocked at the nuclear import step. These results directly associate HIV

integrase and TRN-SR2 for a common role during HIV nuclear import. Representing a significant bottleneck for HIV replication, nuclear import is of utmost importance for successful infection of host cells. The evidence provided supports a mechanism whereby the TRN-SR2/IN interaction directly mediates nuclear import of the viral PIC, validating this protein-protein interaction as a promising target for antiviral therapy.

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Figure 1: $IN^{R263A/K264A}$ has a reduced binding affinity for TRN-SR2 and HIV carrying $IN^{R263A/K264A}$ is replication-defective. (A) Structure of the C-terminal domain of HIV-1 IN based on PDB ID: 1EX4 (61). Amino acids R262, R263 and K264, that are important for the TRN-SR2/IN interaction and reportedly do not affect reverse transcription as single mutants (44) are colored in green. Amino acids K266 and R269, that are important for the TRN-SR2/IN interaction but were reported to negatively affect reverse transcription, are colored in blue. (B) AlphaScreen-based analysis of the outcompetition of the interaction between his_6-tagged WT IN or $IN^{R263A/K264A}$ and GST-TRN-SR2 using untagged IN CTD. IN CTD was titrated out over a fixed concentration of IN (80 nM) and GST-TRN-SR2 (20 nM) and light emission was measured. The graph displays an average of three experiments each performed in duplicate. (C) A representative autoradiogram of an IN 3'-processing assay. The positions of unreacted DNA substrate and 3′-processed products are indicated. In the Graph, the mean DLU/mm²± SD from triplicate samples from two separate experiments is shown, with the WT IN 3'-processing activity set to 100 %. $IN^{R263A/K264A}$ shows 5.1% ± 1.7% 3'-processing activity compared to WT whereas the catalytically dead IN^{D64A} mutant shows no activity (0.3% ± 0.3%). (D) The percentage normalized optical density (OD) of an ELISA measuring strand transfer activity is displayed. The mean OD ± SD from quadruplet samples from three experiments is shown, with the WT IN OD set to 100 %. Both $IN^{R263A/K264A}$ and IN^{D64A} show less than 1.7%± 1.2% strand transfer activity compared to WT. (E) Human PBMCs and were infected with 20,000 pg p24 of HIV NL4-3 WT and HIV NL4-3 $IN^{R263A/K264A}$. Starting three days after infection, supernatants were sampled daily. The mean p24 value ± SD from triplicate samples from one of two representative experiments is shown.

Figure 2: Potent knockdown of importin-α3 does not inhibit HIV infection. (A) A representative western blot shows the levels of importin-α3 (IPOA3) in control cells (shSCR) and importin-α3 knockdown cells (sh93 and sh97) . Anti beta-actin antibody is used as a loading control. (B) HeLaP4 cells depleted of importin-α3 (sh93 and sh97) and control cells (shSCR) were challenged with two dilutions of VSV-G pseudotyped HIV-1 NL4-3 luciferase reporter virus. Three days post infection Fluc activity was measured and normalized to the total amount of protein in the cell lysates. Graphs show the mean values of Fluc light units per µg protein (RLU) ± SD of one representative experiment out of two performed in triplicate.

Figure 3: Replication of HIV NL4-3 $IN^{R263A/K264A}$ is blocked at the stage of nuclear import. For Q-PCR, MT-4 cells were infected with 10^6 pg p24 of HIV NL4-3 WT, $IN^{D64N/D116N/E152Q}$ or $IN^{R263A/K264A}$ virus. At distinct time points after infection, cells were harvested and viral DNA species were quantified by Q-PCR (n = 3, data are represented as mean ± SD). (A and B) Kinetics of late reverse transcripts normalized for RNaseP. (C and D) The number of integrated copies at 48 hours post-infection normalized for RNaseP. (E and F) The number of 2-LTR circles at 24 hours post-infection normalized for RNaseP.

Figure 4: Reduction in nuclear import of HIV NL4-3 IN$^{R263A/K264A}$. Five hours after infection with eGFP-labeled IN virus, HeLaP4 cells were fixed and analyzed by laser-scanning confocal microscopy. The ratio of nuclear/cytoplasmic green PICs was quantified (percentage nuclear PICs). (A) Representative slice of a stack of WT and IN$^{R263A/K264A}$ mutant PIC virus infected cells. The nuclear lamina was immunostained with anti-lamin A/C (red). PICs are identified as green dots and nuclear PICs are highlighted by white arrows. Images are derived from confocal Z-stacks. (B) Cumulative distribution of the percentage of cells containing a certain ratio of nuclear over cytoplasmic PICs. Two independent experiments, with distinct productions of both the WT and the IN$^{R263A/K264A}$ mutant virus, were performed (WT, n = 51 cells; IN$^{R263A/K264A}$, n = 49 cells; p = 0.0054, Mann-Whitney test) (WT, n = 41 cells; IN$^{R263A/K264A}$, n = 44 cells; p = 0.0095, Mann-Whitney test).
(C) Nuclear import was measured in control cells (shSCR) or cells stably depleted for 75 % for TRN-SR2 (shTR3). The cumulative distribution of the percentage of cells containing a certain ratio of nuclear over cytoplasmic PICs is shown forWT virus in shSCR, n = 55 cells, WT in shTR3, n = 49 cells; IN$^{R263A/K264A}$ in shSCR, n = 50 cells and IN$^{R263A/K264A}$ in shTR3, n = 50 cells. Nuclear import of the IN$^{R263A/K264A}$ mutant virus was significantly lower than for WT virus; p = 0.0167, Mann-Whitney test, whereas import of the WT virus in shTR3 cells was not statistically different from that of the IN$^{R263A/K264A}$ mutant virus in shSCR cells; p = 0.5836, Mann-Whitney test.

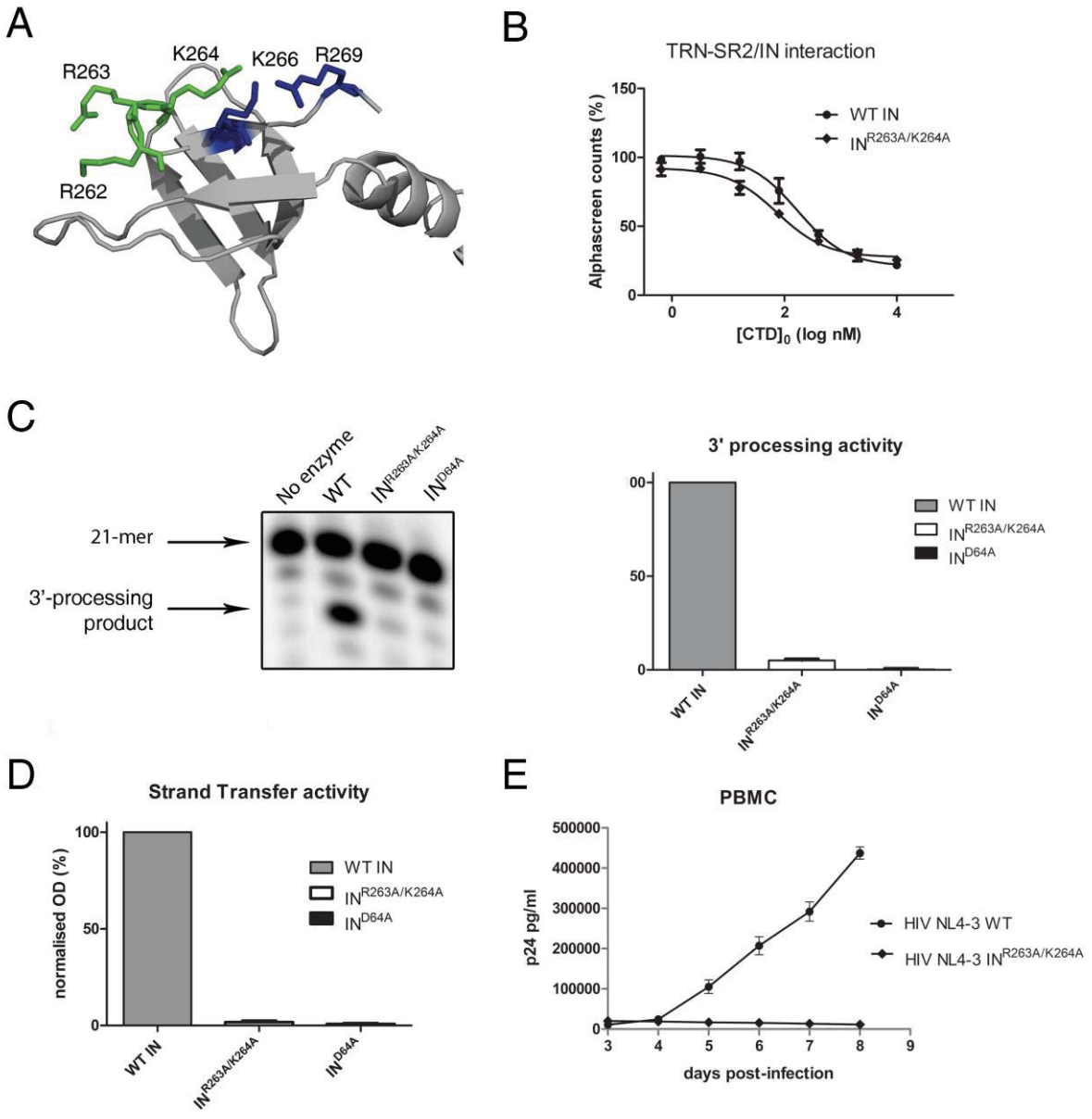
A
R263
K264
K266
R269
R262
B
TRN-SR2/IN interaction
Alphascreen counts (%)
WT IN
IN R263A/K264A
[CTD]0 (log nM)
C
No enzyme
WT
IN R263A/K264A
IN D64A
21-mer
3'-processing product
3' processing activity
WT IN
IN R263A/K264A
IN D64A
D
Strand Transfer activity
normalised OD (%)
WT IN
IN R263A/K264A
IN D64A
E
PBMC
p24 pg/ml
HIV NL4-3 WT
HIV NL4-3 IN R263A/K264A
days post-infection

Figure 1

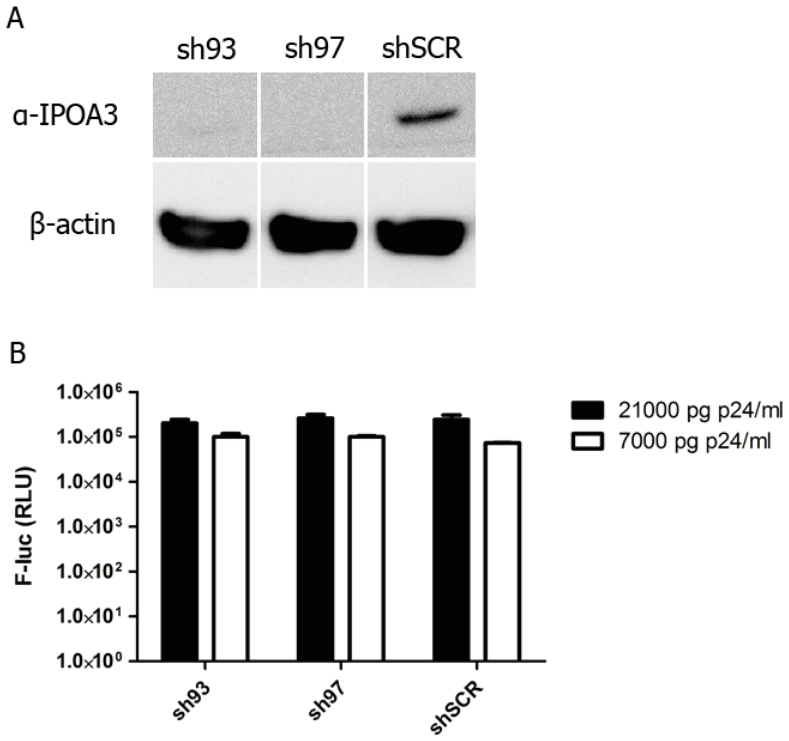
A
sh93
sh97
shSCR
α-IPOA3
β-actin
B
F-luc (RLU)
1.0×10⁶
1.0×10⁵
1.0×10⁴
1.0×10³
1.0×10²
1.0×10¹
1.0×10⁰
sh93
sh97
shSCR
21000 pg p24/ml
7000 pg p24/ml

Figure 2

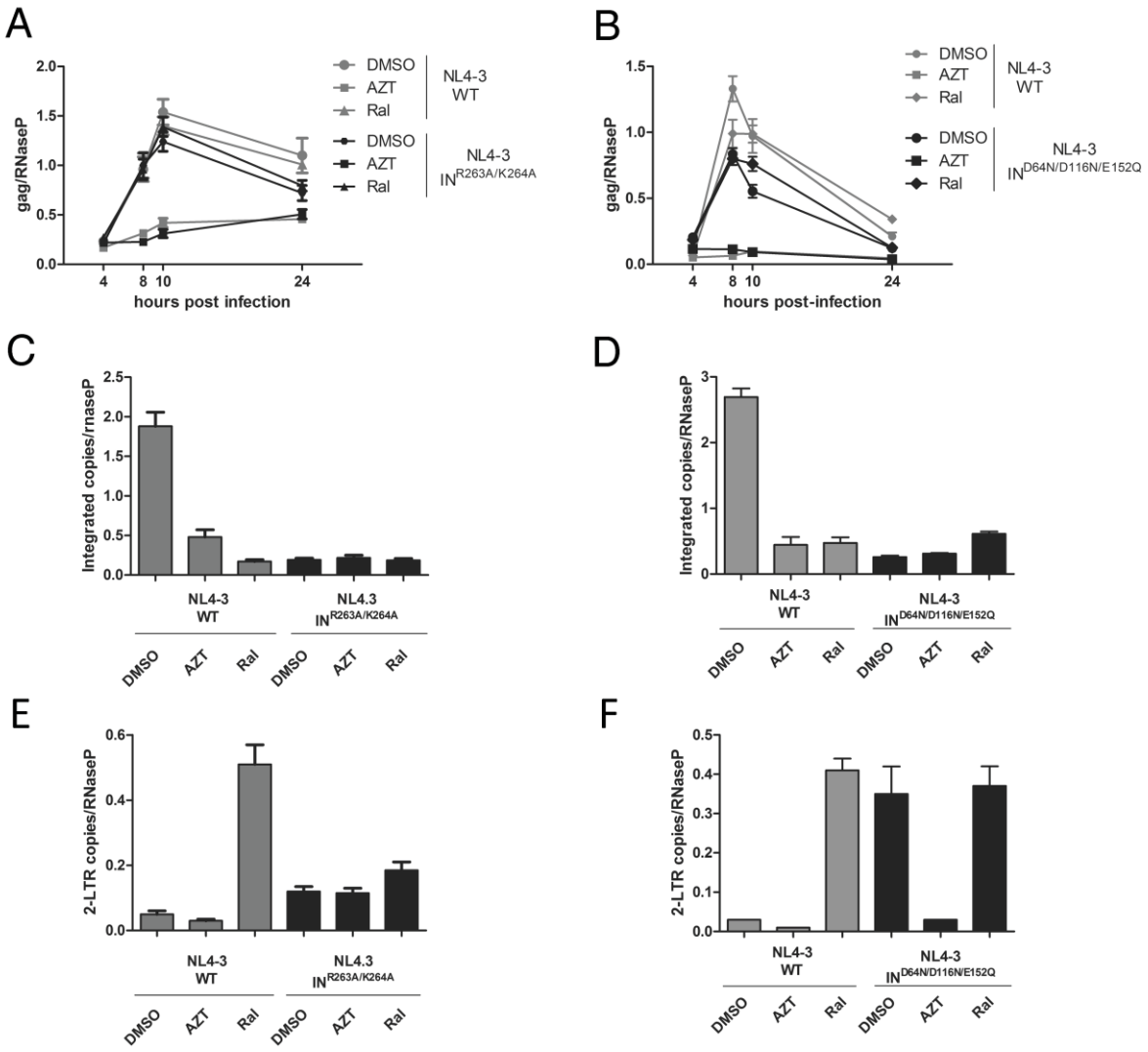
A
gag/RNaseP
hours post infection
DMSO
AZT
Ral
NL4-3 WT
NL4-3 IN R263A/K264A
B
gag/RNaseP
hours post-infection
NL4-3 IN D64N/D116N/E152Q
C
Integrated copies/rnaseP
D
Integrated copies/RNaseP
E
2-LTR copies/RNaseP
F
2-LTR copies/RNaseP

Figure 3

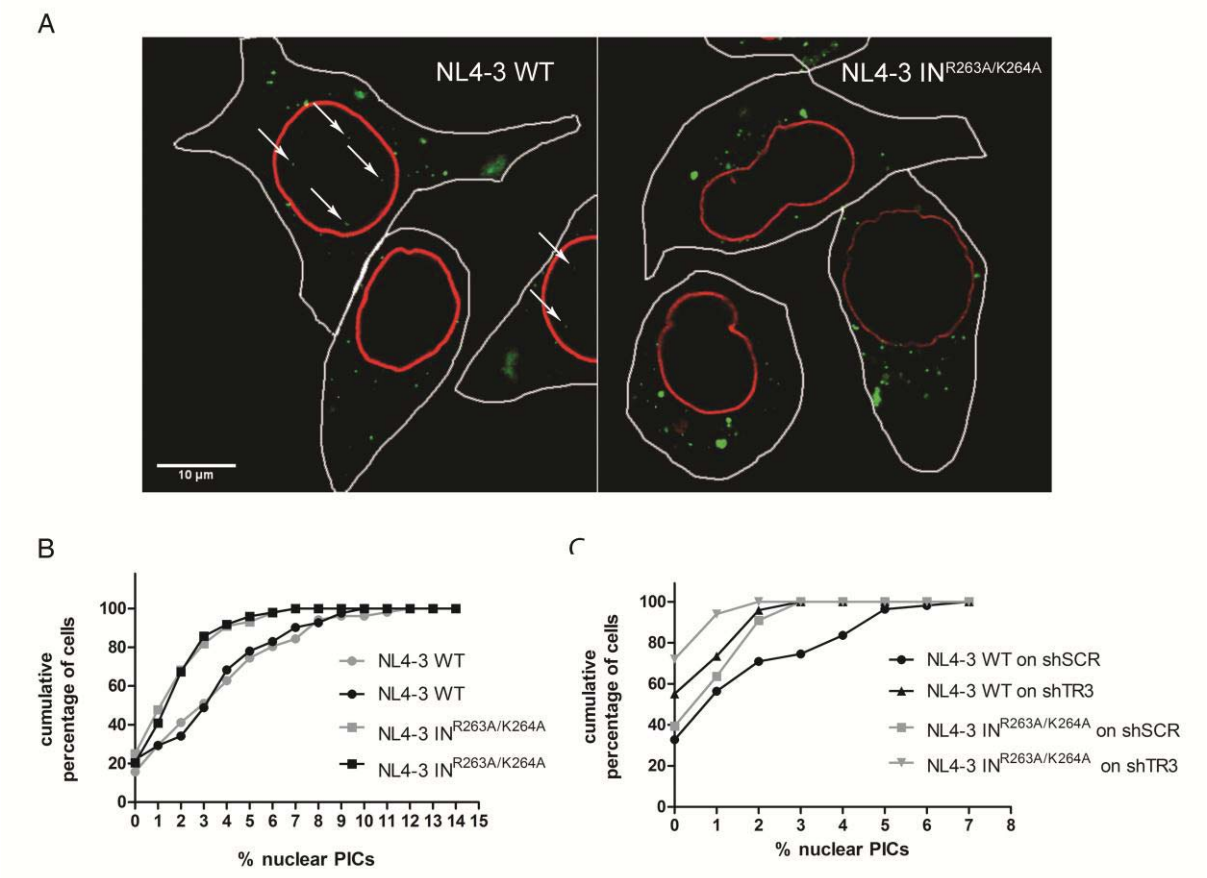
A
NL4-3 WT
NL4-3 IN R263A/K264A
10 µm
B
cumulative percentage of cells
% nuclear PICs
NL4-3 WT
NL4-3 WT
NL4-3 IN R263A/K264A
NL4-3 IN R263A/K264A
C
cumulative percentage of cells
% nuclear PICs
NL4-3 WT on shSCR
NL4-3 WT on shTR3
NL4-3 IN R263A/K264A on shSCR
NL4-3 IN R263A/K264A on shTR3

Figure 4